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TITLE: UV-Induced Triggering of a Biomechanical Initiation Switch Within Collagen Promotes Development of a Melanoma-Permissive Microenvironment in the skin

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14. ABSTRACT
The overall objective of our proposal was to test whether UV irradiation facilitates the exposure of the HU177 cryptic epitopes which may represent a "solid state biomechanical initiation switch" that promotes inflammation, skin damage and the creation of a melanoma permissive niche. Our new studies suggest that activated fibroblasts and mast cells are present within full thickness mouse and human skin and that fibroblasts accumulate in regions rich in basement membrane components after UV exposure. Interestingly, the exposure of subsets of the HU177 epitopes within the basement membrane preparation MatrigelTM depend in part, on the generation of reactive oxygen species. Surprisingly, while cell adhesion to UVB-irradiated MatrigelTM and collagen was higher than that to non-irradiated substrates, migration was significantly inhibited. Moreover, UVB-induced cell adhesion to irradiated substrates was not significantly altered by irradiation of these substrates in the presence of SOD suggesting that UVB-irradiation may cause exposure of a distinct subset of the HU177 epitopes as well as the exposure of additional non-HU177 cryptic sites that play distinct role in modulating cellular behavior. Collectively our new findings provide unique insight into the differential impact of UV irradiated ECM substrates have on the behavior of stromal and inflammatory cells.

15. SUBJECT TERMS
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Table of Contents

Introduction: The overall objective of our project was to test whether UV irradiation facilitates the exposure of the HU177 cryptic collagen epitopes which may represent a "solid state biomechanical initiation switch" that promotes inflammation, skin damage and the creation of a melanoma permissive niche. We have proposed to examine whether specific targeting of the HU177 biomechanical initiation switch prevents or reduces UV-induced inflammation and to determine whether targeting these cryptic collagen sites represent a useful approach to prevent and/or reduce melanoma growth. In particular, we will characterize the kinetics of UV-induced exposure of the HU177 cryptic epitopes in vitro and in vivo using a combination of in vitro biochemical assays, ELISAs and immunohistochemical analysis of UV irradiated-collagen as well as the basement membrane preparation Matrigel^{TM}. In the second aim, we will evaluate the impact of UV-induced alterations in collagen and the basement membrane preparation Matrigel^{TM} has on inflammatory cell, dermal fibroblast, and melanoma cell adhesion, migration, invasion and proliferation as compared to control ECM. Finally, we will determine the biological consequences of UV-induced exposure of the HU177 cryptic epitope on inflammatory cell infiltration and on the ability of melanoma cells to establish tumors in vivo.

Body: Due in part to changing to a new and more precise and efficient UV-irradiator; we were slowed down in terms of completing all of the original proposed aims. In this regard, we were granted a non-cost extension to allow time to finish our project. Despite this delay we have made significant progress towards the overall goals of our project during the third funding period (September 2012 through September 2013). Specifically, we have made substantial progress on all specific aims 1-3 and their associated tasks. A detailed summary of the research accomplishments as they pertain to the tasks outlined in the statement of work is provided below. Our prior studies have indicated that UVA and UVB irradiation can dose dependently induce conformational changes in both collagen type-I and collagen type-IV resulting in the exposure of the HU177 cryptic collagen epitopes. Importantly, the relative level of exposure of the HU177 epitopes as indicated by ELISA, varied depending on the collagen type, wavelength and dose of UV irradiation. In addition, our previous studies indicated that human melanoma cell and fibroblast adhesion was generally enhanced on UV irradiated collagen. Interestingly, the adhesive and migratory behavior of inflammatory cells such as macrophages on UV irradiated collagen was also differentially altered suggesting a complex cell type and collagen specific response to distinct UV wavebands in vitro. These novel findings are consistent with the possibility that unique physical alterations in the three-dimensional structure of ECM proteins may represent a mechanism to initiate triggering of a biomechanical switch that allows multiple cell types within the tissue microenvironment to differentially interact with cryptic epitopes thereby altering their behavior. Given our exciting new data suggesting that mast cell behavior in vivo may be modulated by interactions with UV-irradiated ECM proteins, we have added a new murine transformed mast cell line (P815) to our studies as a cell model of how cellular interactions with UV-irradiated ECM proteins may modulate inflammation and melanoma tumor initiation and progression.

Summary of research accomplishments

UVB-irradiation of mouse skin is associated with altered αSMA expressing fibroblast distribution. Our previous studies suggested that structural changes induced by UVB-irradiation (310nm) of collagen differentially alter human fibroblast behavior in vitro. Alpha smooth muscle actin (αSMA) is a well-known marker of activated fibroblasts and activated fibroblasts are thought to contribute to ECM remodeling during tumor development. Given our previous observations with human fibroblasts we first examined whether the human fibroblasts used in our studies expressed αSMA, a known marker of an activated phenotype. As shown in figure 1A the in vitro cultured human dermal fibroblast used in our studies indeed express αSMA confirming their activated phenotype. Given these results we next examined the relative distribution of αSMA expressing fibroblast in mouse skin before and after UV irradiation. As shown in figure 1B, irradiation of full thickness mouse skin with a single dose of UVB (0.1J/cm^2) resulted in alteration in the distribution of αSMA expressing fibroblast 24 hours later. UVB-irradiated skin exhibited enhanced levels of αSMA expressing fibroblasts within the lower regions of the dermis and appeared to be enriched around basement membrane

associated structures. These data suggest that UVB may alter the distribution of αSMA expressing fibroblast that have the capacity to alter ECM structure.

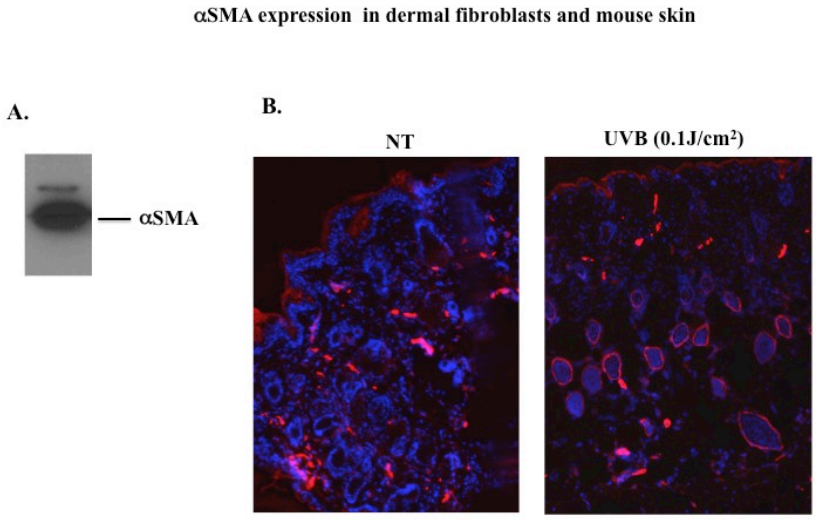


Figure 1. UVB-irradiation of mouse skin is associated with altered distribution of αSMA expressing fibroblast. A). Western blot analysis if human dermal fibroblasts for expression of αSMA. B) Murine skin from non-treated (NT) or UVB irradiated (0.1J/cm^2) animals stained for expression of αSMA (Red). Dapi stain indicated in blue.

Reduced migration of human dermal fibroblast following UVB-irradiation of Matrigel™. Given the differential distribution of fibroblast following UVB irradiation of full thickness skin and our previous results indicating altered adhesive behavior, we examined the impact of UVB irradiated Matrigel™ has on fibroblast migration. As shown in figure 2, a distinct reduction in dermal fibroblast migration on Matrigel™ was observed with all doses of UVB irradiation as compared to untreated Matrigel™. These data suggest that the structural changes induced within Matrigel™ by UVB irradiation reduces the capacity of dermal fibroblast to migrate under these conditions.

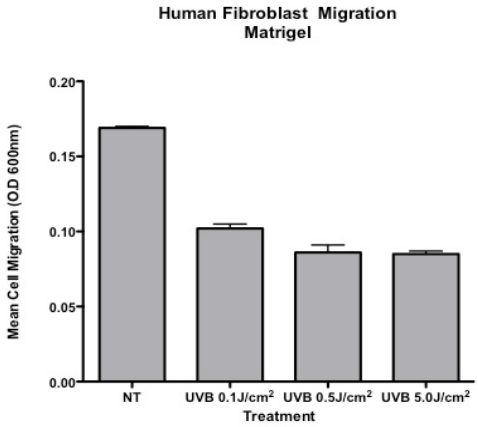


Figure 2. Reduced migration of dermal fibroblast on UVB-irradiation of Matrigel™. Matrigel™ was either non-treated (NT) or irradiated with UVB (310nm) over a dose range (0-5.0J/cm^2). Matrigel™ was coated (5.0ug/ml) on membranes from transwell inserts and blocked with BSA. Human dermal fibroblasts were seeded on the wells and allowed to migrate. Data bars represent mean cell migration (Optical Density O.D) ± standard deviations from triplicate wells.

UVB-irradiation of Matrigel™ enhances exposure of the HU177 cryptic epitope. Given our new data and previous observations, we examined the impact of UVB irradiation of Matrigel™ on the exposure of the HU177 epitopes using two distinct antibodies including Mab HU177 and the humanized Mab D93 directed to distinct subsets of the PGxPG containing HU177 cryptic collagen epitopes. As shown in figure 3A and B, UVB irradiation of Matrigel™ with a single dose (0.1J/cm^2) resulted in enhanced exposure of the HU177 epitopes. Importantly, irradiation of Matrigel™ in the presence of 25units/ml of the reactive oxygen species (ROS) scavenger Superoxide Dismutase (SOD) differentially reduced exposure of the HU177 epitopes. These important results suggest for the first time that the exposure of distinct subsets of the PGxPG containing

HU177 cryptic collagen epitopes within the ECM preparation Matrigel™ is complex and depends in part on the generation of ROS.

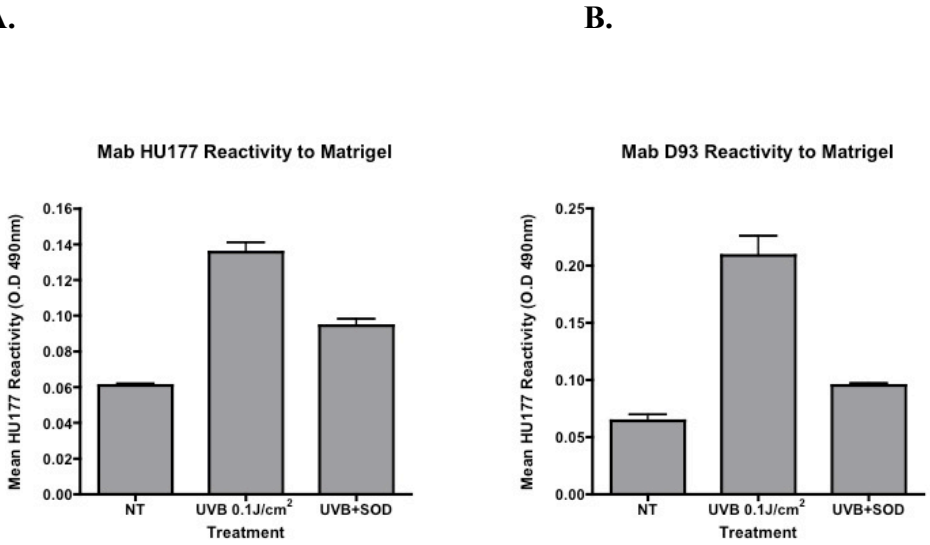


Figure 3. UVB-irradiation of Matrigel™ enhances exposure of the HU177 cryptic collagen epitope. Matrigel™ was either not treated (NT) or irradiated with UVB (310nm) at dose of 0.1J/cm^2 in the presence or absence of SOD. Wells were coated and solid phase ELISAs was carried out to assess exposure of the PGxPG containing collagen epitopes in Matrigel™. (A) Reactivity of Mab HU177. (B) Reactivity of Mab D93. Data bars represent mean reactivity of the indicated antibodies (Optical Density O.D) ± standard deviations from triplicate wells.

Fibroblast adhesion to Matrigel™ irradiated in the presence or absence of SOD. Our previous studies suggest a differential requirement for reactive oxygen species (ROS) for the exposure of subsets of the HU177 cryptic collagen epitope within Matrigel™. To examine whether fibroblast adhesion was altered on Matrigel™ irradiated with UVB in the presence or absence of SOD, in vitro adhesion assays were performed. As shown in figure 4, while a single dose (0.5J/cm^2) of UVB irradiation of Matrigel™ caused a small enhancement of fibroblast adhesion, this increased adhesion was decreased on UVB irradiated Matrigel™ in the presence of SOD. However, given the limited changes observed, additional experiments are required to confirm the significance of these observations. These data suggest that the generation of ROS may play a role in regulating the exposure of a cryptic site in Matrigel™.

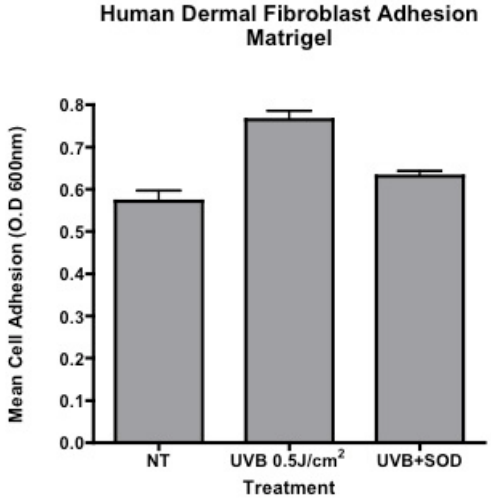


Figure 4. Fibroblast adhesion to Matrigel™ irradiated in the presence or absence of SOD. Matrigel™ was either not treated (NT) or irradiated with UVB (310nm) (0.5J/cm^2) in the presence or absence of 25units/ml of SOD. Microtiter wells were coated (5.0ug/ml) with untreated or UVB irradiated Matrigel™ and human dermal fibroblasts were seeded in wells and allowed to attach. Data bars represent mean cell adhesion (Optical Density O.D) ± standard deviations from triplicate wells.

Fibroblast adhesion to collagen irradiated in the presence or absence of SOD. Our previous studies suggest a differential requirement for reactive oxygen species (ROS) for the exposure of subsets of the HU177 cryptic collagen epitope within Matrigel™. To examine whether fibroblast adhesion to collagen is altered following UVB irradiation of collagen in the presence or absence of SOD, in vitro adhesion assays were

performed. As shown in figure 5A, a single dose (0.5J/cm^2) of UVB irradiation of collagen type-I caused a near two-fold increase in fibroblast adhesion. Interestingly, little if any change in fibroblast adhesion to UVB irradiated collagen type-I in the presence of SOD was observed. In similar studies, while little change in fibroblast adhesion was observed between non-irradiated and UVB irradiated collagen type-IV, fibroblast adhesion to collagen type-IV irradiated in the presence of SOD was not effected (figure 5B). Taken together, these studies suggest that the generation of ROS during UVB irradiation of collagen to expose distinct cryptic sites exhibit little capacity to alter fibroblast adhesion under these experimental conditions.

A. **B.**

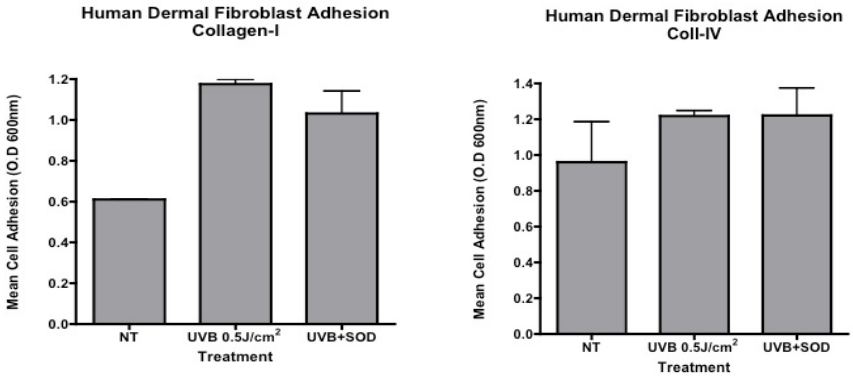


Figure 5. Fibroblast adhesion to collagen irradiated in the presence or absence of SOD. Collagen type-I (A) and collagen type-IV (B) was either not treated (NT) or irradiated with UVB (310nm) (0.5J/cm^2) in the presence or absence of 25units/ml of SOD. Microtiter wells were coated (5.0ug/ml) with untreated or UVB irradiated collagen and human dermal fibroblasts were seeded in wells and allowed to attach. Data bars represent mean cell adhesion (Optical Density O.D) $\pm$ standard deviations from triplicate wells.

Dose dependent enhancement of mast cell adhesion to UVB-irradiation of Matrigel^{TM}. Given previous findings suggesting the differential distribution of inflammatory cells including macrophages and neutrophils following UVB irradiation of full thickness skin and our previous results indicating altered accumulation of mast cells within UVB irradiated skin which could be inhibited by function blocking antibody D93 directed to a subset of the HU177 cryptic collagen epitopes, we examined the impact of UVB irradiated Matrigel^{TM} has on a transformed murine mast cell line (P815). As shown in figure 6, a dose dependent increase in mast cell adhesion was observed to Matrigel^{TM} irradiated with increasing doses of UVB. These data suggest for the first time that unique changes induced by UVB irradiation may enhances the ability of mast cells to attach to Matrigel^{TM}.

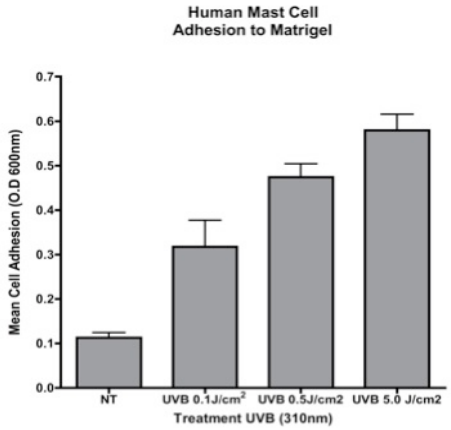


Figure 6. Dose dependent enhancement of mast cell adhesion to UVB-irradiated of Matrigel^{TM}
.Matrigel^{TM} was either non-treated (NT) or irradiated with UVB (310) over a dose range (0-5.0J/cm^2). Matrigel^{TM} was coated (5.0ug/ml) on microtiter wells and blocked with BSA. Murine transformed mast cells (P815) were seeded on the wells and allowed to attach. Data bars represent mean cell adhesion (Optical Density O.D) $\pm$ standard deviations from triplicate wells.

Reduced migration of mast cells on UVB-irradiated Matrigel^{TM}. Given the altered adhesion of mast cells to irradiated Matrigel^{TM} and the altered accumulation of mast cells in full thickness skin following UVB irradiation, we examined the impact of UVB irradiated Matrigel^{TM} had on mast cell migration. As shown in

figure 7, surprisingly a sharp reduction in mast cell migration was observed on UVB-irradiated Matrigel™ at all doses tested as compared to untreated Matrigel™. These surprising findings suggest that the while the changes induced within Matrigel™ by UVB irradiation dose dependently enhance mast cell adhesion, these alterations have the opposite effect on mast cell migration, indicating a complex role for distinct changes in the ECM components of Matrigel™ in governing mast cell behavior in vitro.

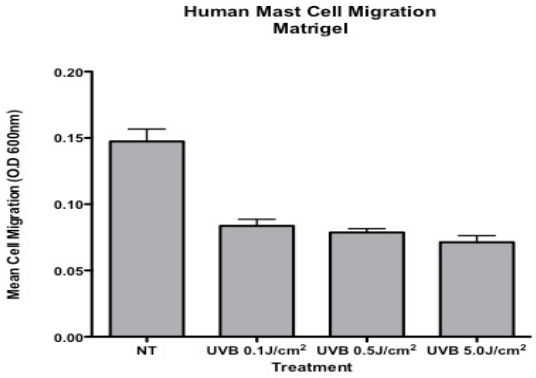
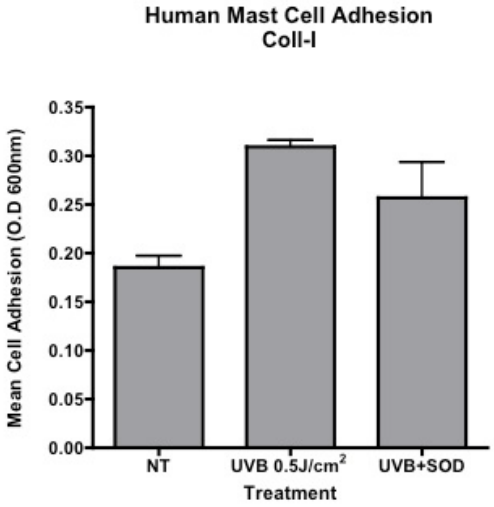


Figure 7. Reduced mast cell migration on UVB-irradiated Matrigel™. Matrigel™ was either non-treated (NT) or irradiated with UVB (310) over a dose range (0-5.0J/cm^2). Matrigel™ was coated (5.0ug/ml) on membranes from transwell inserts and blocked with BSA. Transformed murine mast cells (P815) were seeded in the upper chamber and allowed to migrate for 4hrs. Data bars represent mean cell migration (Optical Density O.D) ± standard deviations from triplicate wells.

Mast cell adhesion to collagen irradiated in the presence or absence of SOD. Our previous studies suggest a differential requirement for reactive oxygen species (ROS) for the exposure of a subset of the HU177 cryptic collagen epitopes within Matrigel™ and a differential impact on fibroblast adhesion to collagen. To examine whether mast cell adhesion may be altered on UVB irradiated collagen in the presence or absence of SOD, in vitro adhesion assays were performed. As shown in figure 8A, a single dose (0.5J/cm^2) of UVB irradiation of collagen type-I caused a nearly 50% increase in mast cell adhesion to collagen type-I as compared to non-irradiated collagen type-I. Interestingly, little if any change in mast cell adhesion to collagen type-I irradiated with UVB in the presence of SOD was observed. In similar studies little change in mast cell adhesion was observed between non-irradiated and UVB irradiated collagen type-IV. Moreover, mast cell adhesion to collagen type-IV irradiated in the presence of SOD was also not effected (figure 8B). Taken together, these studies suggest that the generation of ROS during UVB irradiation of collagen to expose distinct cryptic sites exhibit little capacity to alter mast cell adhesion under these experimental conditions.

A.



B.

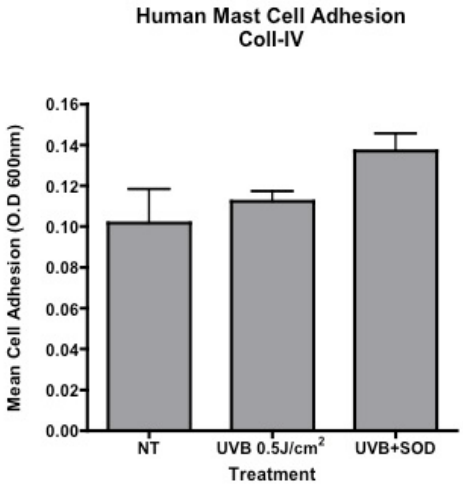


Figure 8. Mast cell adhesion to collagen irradiated in the presence or absence of SOD. Collagen type-I (A) and collagen type-IV (B) was either not treated (NT) or irradiated with UVB (310nm) (0.5J/cm^2) in the presence or absence of 25units/ml of SOD. Microtiter wells were coated (5.0ug/ml) with untreated or UVB irradiated collagen and mast cells (P815) were seeded in wells and allowed to attach. Data bars represent mean cell adhesion (Optical Density O.D) ± standard deviations from triplicate wells.

Detection of CD163 positive macrophages in full thickness human skin. Our previous studies have indicated that the HU177 cryptic collagen epitopes can be detected in full thickness human foreskin. To establish the experimental conditions for detecting changes in the relative accumulation of human macrophages following UV irradiation of full thickness human skin, we first examined the base line levels of human macrophages using a antibodies directed to the well –established monocyte/macrophage marker CD163. As shown in figure 9, CD163 expressing human macrophages (Red) are present at a basal level within explanted human neonatal full thickness foreskin. Importantly, macrophages can be detected in both non-irradiated as well as UVB (310nm) irradiated (0.1J/cm^2) explanted human skin. These data confirm the utility of the anti-CD163 antibody for the detection of human macrophages in frozen sections of explanted human foreskin that will be used in our human/mouse chimeric model.

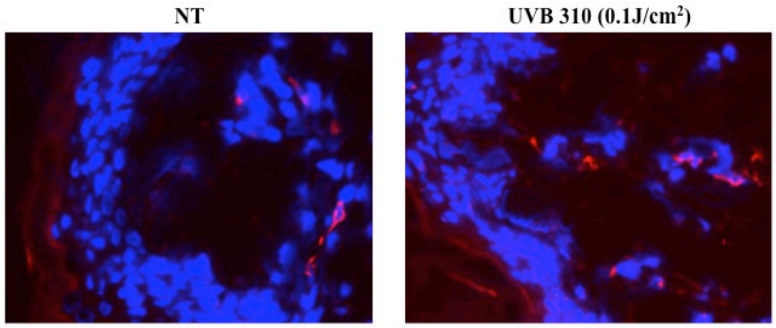


Figure 9. Detection of CD163 positive macrophages in full thickness human skin. Fresh human neonatal foreskin obtained 24hrs following surgical resection was either not treated (NT) or irradiated ex vivo (0.1J/cm^2) with UVB (310nm). Full thickness human skin was embedded and tissues sections prepared. Tissues were stained with antibodies directed to the human monocyte/macrophage marker CD163. Representative examples of human skin stained with anti-CD163 antibody. Red indicates CD163 expressing macrophages. Photos taken at a magnification of 400x.

Detection of αSMA expressing fibroblasts and mast cell tryptase in full thickness human skin. Our previous studies have indicated a possible redistribution of αSMA expressing fibroblasts in mouse skin following UV irradiation. In addition, an alteration in accumulation of mast cells in mouse skin was also observed. To establish the experimental conditions for detecting changes in αSMA expressing fibroblast and mast cells following UV irradiation of full thickness human skin, we examined the base line levels of αSMA (A) and mast cell tryptase (B) using antibodies directed to these well –established markers of activated fibroblasts and mast cells respectively. As shown in figure 10A, αSMA-expressing fibroblasts (Red) could be easily detected within the dermal regions of untreated or UVB-irradiated explanted full thickness human skin. In similar studies, mast cell tryptase (Red) was also readily detected within the dermal regions of untreated or UVB-irradiated explanted human skin, thereby confirming the basal levels of these markers (Figure 10B).

A. **B.**

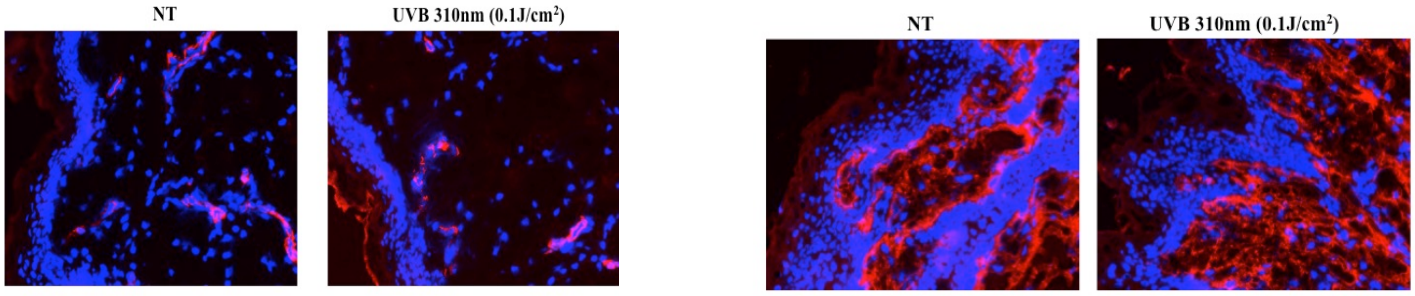


Figure 10. Detection of αSMA expressing fibroblasts and mast cell tryptase in full thickness human skin. Fresh human neonatal foreskin obtained 24hrs following surgical resection was either not treated (NT) or irradiated ex vivo (0.1J/cm^2) with UVB (310nm). Full thickness human skin was embedded and tissues sections prepared. Tissues were stained with antibodies directed to αSMA (A) and mast cell tryptase (B). Photos taken at a magnification of 200x.

Key Research Accomplishments:

1). UVB- irradiation of full thickness mouse skin in vivo alters distribution of αSMA expressing fibroblasts.

2). UVB irradiation of Matrigel™ reduces the migratory behavior of fibroblast on this substrate in vitro.

3). SOD differentially inhibits UVB-mediated exposure of the HU177 cryptic epitopes within Matrigel™.

4). Fibroblasts exhibit little change in attachment to UVB-irradiated Matrigel™ in the presence or absence of SOD.

5). Fibroblasts exhibit little change in attachment to UVB-irradiated collagen in the presence or absence of SOD.

6). UVB-irradiation of Matrigel™ dose dependently enhances mast cell adhesion to this substrate.

7). UVB-irradiation of Matrigel™ reduces mast cell migration on this substrate.

8). Mast cells exhibit little change in attachment to UVB-irradiated collagen in the presence or absence of SOD.

9). Basal levels of CD163 expressing human macrophages are detected within untreated and UVB-irradiated full thickness human skin.

10). Basal levels of αSMA expressing fibroblast and mast cell tryptase are detected within untreated and UVB-irradiated full thickness human skin.

Reportable Outcomes: None

Conclusions:

Our previous experimental findings indicate that irradiation with UVA and UVB wavebands results in unique and differential structural changes in collagen type-I, collagen type-IV and the ECM preparation Matrigel™ in vitro leading to the differential exposure of the HU177 cryptic collagen epitopes. Importantly, our studies have revealed that the extent of the exposure of the HU177 cryptic epitopes within each of the distinct ECM molecules including collagen type-I collagen type-IV and Matrigel™ in vitro varies depending on the particular waveband (UVA/UVB) and exposure dose of irradiation. Expanding on our previous experimental findings, our current studies indicate that activated fibroblasts that exhibit a myofibroblast-like phenotype (expression of αSMA) are present within the dermal regions of full thickness mouse and human skin. Interestingly, a distinct redistribution of αSMA expressing fibroblast can be detected 24 hours after exposure of mouse skin to a single UVB irradiation dose of $0.1J/cm^2$. The redistributed αSMA positive fibroblasts were often observed accumulating in regions that appeared to be rich in basement membrane components. In this regard, our new findings indicate that $0.1J/cm^2$ dose of UVB irradiation can enhance exposure of the HU177 epitope within the basement membrane preparation Matrigel™. Interestingly, the exposure of distinct subsets of the PGxPG containing HU177 cryptic epitope within Matrigel™ appears to be dependent in part, on the generation of free radical reactive oxygen species as the ROS scavenger superoxide dismutase (SOD) differentially inhibited exposure of these cryptic sites in vitro. Surprisingly, while fibroblast adhesion to UVB-irradiated Matrigel™ was found to be higher than that to non-irradiated Matrigel™, fibroblast migration was significantly inhibited. Moreover, the UVB-induced fibroblast adhesion to UVB-irradiated Matrigel™ was not significantly altered by irradiation of Matrigel™ in the presence of SOD. Given that a subset of the HU177 cryptic epitopes exposed within Matrigel™ are dependent in part on generation of

free radicals, our new findings are consistent with the possibility that UVB-irradiation may cause exposure of a subset of the HU177 epitopes as well as exposure of additional non-HU177 cryptic sites that play distinct role in modulating fibroblast behavior. In addition, our new data also indicates that the ability of fibroblasts to attach to cryptic epitopes within collagen type-I or collagen type-IV is not significantly altered by UVB-irradiation in the presence of SOD. Collectively our new data suggest that the UVB-induced cryptic epitopes that appear to enhancing fibroblast cell adhesion to either Matrigel™ or collagen are not altered functionally following irradiation in the presence by SOD.

Given our previous studies indicating that the HU177 epitope appear to play a functional role in regulating the accumulation of mast cells within full thickness murine skin following UVB-irradiation, we sought to assess the impact of UVB irradiation of Matrigel™ and collagen in the presence or absence of SOD might have on mast cell adhesion to these ECM substrates. Our current studies indicate for the first time that UVB-irradiation of Matrigel™ results in a dose dependent increase in mast cell adhesion as compared to control. Interestingly, as was observed with fibroblasts, irradiation of Matrigel™ with UVB wavebands resulted in a distinct reduction in mast cell migration. These data suggest that UVB-irradiation of Matrigel™ can result in unique changes that differentially alter mast cell behavior in vitro. Moreover, as was observed in fibroblasts, mast cell adhesion to UVB-irradiated collagen was not significantly altered by irradiation of collagen in the presence of SOD suggesting that the generation of ROS during irradiation had little direct impact on adhesive functions of the cryptic epitopes exposed.

Finally, given our in vitro and in vivo studies implicating functional roles for inflammatory cells such as macrophages and mast cell as well as activated αSMA-expressing fibroblasts in the UV-mediated inflammatory response in full thickness skin, we examined the basal levels of macrophages, fibroblast and mast cells within explants of full thickness human skin. Importantly, our new studies have indicated that antibodies directed to CD163, αSMA and mast cell tryptase allows us to detect the relative levels of these cell types within human skin in our human/mouse chimeric model. Taken together, we have made substantial progress towards the overall goals of our proposal as indicated by the progress on specific experiments detailed in aim 1, tasks 1-3, specific aim 2 tasks 1-3 and specific aim 3 task 1. In conclusion, our current findings suggest that not only can UV-irradiation of specific ECM proteins differentially alter the behavior of both fibroblasts and mast cells on specific ECM substrates in vitro but that UVB-radiation may generate new previously unknown regulatory sites used by these cell types in vitro. In addition, our new finding also provide critical insight into the basal distribution of human macrophages, mast cells and fibroblasts within full thickness human skin that will be used in our human/mouse chimeric model.

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Appendices: none